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Safety and immunogenicity of a plant-derived rotavirus-like particle vaccine in adults, toddlers and infants

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ABSTRACT

Background: This study is the first clinical trial for a parenteral non-replicating rotavirus vaccine developed using virus-like particle (VLP) technology.
Methods: This open-labeled, randomized, placebo-controlled trial was conducted in two parts: Part A (a first-in-human study in Australian adults) and Part B (ascending dose and descending age in South African adults, toddlers and infants). In Part A, two cohorts of 10 adults were assigned to receive a single intramuscular injection of 1 of 2 escalating dose levels of the rotavirus VLP (Ro-VLP) vaccine (7 μg or 21 μg) or placebo. In Part B, one cohort of 10 adults was assigned to receive a single injection of the Ro-VLP vaccine (21 μg) or placebo, two cohorts of 10 toddlers were assigned to receive 2 injections of 1 of 2 escalating dose levels of the Ro-VLP vaccine (7 μg or 21 μg) or placebo 28 days apart, and three cohorts of 20 infants were assigned to receive 3 injections of 1 of 3 escalating dose levels of the Ro-VLP vaccine (2.5 μg, 7 μg or 21 μg) or placebo or 2 doses of oral Rotarix 28 days apart. Safety, reactogenicity and immunogenicity were assessed.
Results: There were no safety or tolerability concerns after administration of the Ro-VLP vaccine. The Ro-VLP vaccine induced an anti-G1P[8] IgG response in infants 4 weeks after the second and third doses. Neutralizing antibody responses against homologous G1P[8] rotavirus were higher in all Ro-VLP infant groups than in the placebo group 4 weeks after the third dose. No heterotypic immunity was elicited by the Ro-VLP vaccine.
Conclusions: The Ro-VLP vaccine was well tolerated and induced a homotypic immune response in infants, suggesting that this technology platform is a favorable approach for a parenteral non-replicating rotavirus vaccine.
Clinical Trial Registration: NCT03507738.

1. Introduction

Rotavirus is the leading cause of diarrhea deaths in infants and young children [1]. Almost all children are infected before the age of 5 years, regardless of where they live. Rotavirus also causes considerable morbidity, with global estimates of around 2 million hospitalizations and around 20 million outpatient visits annually among children aged < 5 years, and is responsible for around 40% of acute gastroenteritis hospitalizations among children aged < 5 years in regions with no widespread rotavirus vaccine use [2–4].

Rotavirus demonstrates a complex architecture of three protein layers surrounding 11 segments of double-stranded RNA. The inner

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and intermediate protein layers are composed of viral protein (VP) 2 and VP6, respectively, and the outer layer is composed of VP7 with VP4 spikes [5]. Rotavirus genotypes are classified according to VP7 and VP4 which induce neutralizing antibodies with VP7 specifying the G genotype and VP4 specifying the P genotype [6]. The six genotypes - G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] - are responsible for a majority of infections in most of the world [7].

In 2009, the World Health Organization (WHO) recommended the inclusion of rotavirus vaccines into the national immunization programs of all countries [8]. Vaccination has been demonstrated to be effective in reducing the global burden of rotavirus gastroenteritis, with all-cause hospitalizations due to diarrhea in children aged <5 years decreasing by 38%, rotavirus disease-associated hospitalizations decreasing by 67% and all-cause diarrhea deaths decreasing by 42% during the first 10 years after the implementation of routine rotavirus vaccinations in national immunization programs [9].

Four live oral rotavirus vaccines have so far been prequalified by the WHO: RotaTeq (a pentavalent human-bovine reassortant vaccine; Merck & Co., Inc., West Point, PA, USA), Rotarix (a human monovalent G1P[8] vaccine; GlaxoSmithKline Biologicals, Rixensart, Belgium), Rotavac (a human monovalent G9P[11] vaccine; Bharat Biotech International Ltd., Hyderabad, India) and Rotasiil (a lyophilized pentavalent human-bovine reassortant vaccine; Serum Institute of India PVT. LTD., Pune, India) [10]. Among them, RotaTeq and Rotarix are commercially available worldwide. Both vaccines have proven to be highly effective against severe gastroenteritis in high-income countries (80–100%) [11,12], although they have shown lower efficacy in low- to middle-income countries (LMIC) (approximately 50%) [13–15]. It is hypothesized that interference by high titers of rotavirus antibodies acquired transplacentally, micronutrient deficiency, malnutrition, interfering gut microflora, enteric co-infections, concomitant disease and differences in rotavirus epidemiology might contribute to the sub-optimal performance in LMIC [16]. Furthermore, oral rotavirus vaccines have a risk of intussusception, which is believed to be triggered by replication of the attenuated virus. The incidence of rotavirus vaccine-related intussusception is 1 to 6 excess cases per 100000 infants vaccinated [17].

As an alternative strategy to current live-attenuated vaccines, several non-replicating parenteral rotavirus vaccines are under development [18]. Various approaches have been taken to produce non-replicating parenteral vaccines including inactivated rotavirus particles, protein subunits or virus-like particles (VLPs) that have a structure similar to live virus [19]. Producing VLPs is a well-established approach for developing non-replicating parenteral vaccines [20,21]. Plant-based technology for the production of VLPs has been in development for more than 25 years. Past studies have demonstrated that plants can produce VLPs from various viral origins such as hepatitis B virus core-like particles, papillomaviruses and influenza virus and with various structural characteristics (e.g., enveloped displaying target antigens, non-enveloped capsid-type, etc.) [22–24]. When administered properly, plant-derived influenza VLP vaccines can induce protective immune responses in animal models [25], and cross-reactive antibody and T cell response in humans [26]. To date, rotavirus-like particles have been produced using several expression strategies [27], but no rotavirus VLPs have been tested in humans.

Ro-VLP is a monovalent (G1 type) non-replicating, rotavirus-like particle composed of VP7, VP6 and VP2 surface antigens, and this vaccine candidate has been shown to induce antibody responses without any significant toxicity when parenterally administered to rats and rabbits [28]. In this clinical trial, which includes a first-in human study, we investigated the safety, reactogenicity and immunogenicity of the Ro-VLP vaccine at different doses in healthy adults, toddlers and infants.

2. Methods

2.1. Ro-VLP production

Ro-VLP was produced in *Nicotiana benthamiana* using the *Agrobacterium* infiltration-based transient recombinant expression platform by Medicago Inc. (Québec, QC, Canada) as previously described [28]. Ro-VLP was obtained by recombinant expression of the VP7, VP6 and VP2 surface proteins along with NSP4 non-structural helper protein that interact together to form the VLPs, and are therefore devoid of viral genetic material. Substantial increase in Ro-VLP yield has been confirmed by adding the DNA construct of NSP4. The gene sequences of VP7 and VP6/2 used for recombinant expression are derived from the human rotavirus RIX4414 (Rotarix) strain and Wa strain (G1P[8]), respectively. The *Agrobacterium* inoculum was infiltrated into plant biomass using a vacuum chamber, and the plants were then incubated for 8 days in controlled expression chambers. Following harvest, the leaves and petioles were mechanically diced, and the resultant biomass was processed through a series of clarification and purification steps. Subsequently, a stabilizer and a cryoprotectant were added to the purified Ro-VLP drug substance (DS) prior to sterilizing grade filtration. Ro-VLP DS was filled in sterile bags and stored frozen at ≤ -65 °C protected from light until ready for formulation and filling. These procedures were performed in Medicago's facility under Good Manufacturing Practices (GMP) appropriate for clinical studies. Ro-VLP DS (lot number.: ABXBN01-180011) is a sterile, transparent to opalescent colorless to greenish liquid suspension consisting of Ro-VLPs in a Tris-based formulation buffer, pH 7.4. Ro-VLP is composed of 11% VP7, 49% VP6 and 39% VP2, assembled in VLP of 87 nm in size [28].

2.2. Formulation of Ro-VLP vaccine

The United States Pharmacopeia (USP) Type I single dose glass vials filled with 0.5 mL of Ro-VLP at a concentration of 10 μg/mL, 28 μg/mL or 84 μg/mL, and the glass vials filled with 1.0 mL of 0.4% aluminum hydroxide were manufactured by Medicago Inc. under GMP. Vials of Ro-VLP and aluminum hydroxide were stored at ≤ -65 °C and room temperature, respectively, until the time of preparation by the pharmacist. Using a syringe, 0.5 mL of 0.4% aluminum hydroxide was injected into vials containing Ro-VLP. The vials were then swirled until a homogenous mixture was obtained. A total of 0.5 mL of the mixture (2.5 μg, 7 μg, or 21 μg of Ro-VLP containing 0.2% aluminum hydroxide) was drawn into a syringe and administered intramuscularly to subjects in the Ro-VLP vaccine groups within 5–60 min after mixing. Rotarix (GlaxoSmithKline, Rixensart, Belgium) was sourced at the study site and administered orally to infants in the Rotarix group according to the manufacturer's instructions. Sterile saline 0.9% for injection was sourced as single use containers by each study site. Each 0.5 mL dose was drawn into a syringe and administered intramuscularly to subjects in the placebo group.

2.3. Study design

This phase 1, randomized, open-labeled, placebo-controlled, descending age, dose escalation study consisted of two parts: Part

A, the first-in human-study, and Part B, the dose-escalation phase initiated in adults, followed by toddlers, and then infants.

Part A was conducted at a single site (CMAX Clinical Research Pty. Ltd., Adelaide) in Australia. A total of 20 healthy adults were enrolled and 2 cohorts of 10 adults aged 18–35 years were randomly assigned to receive a single intramuscular injection of 1 of 2 escalating doses of the Ro-VLP vaccine (7 μg or 21 μg) or placebo. Each adult dose cohort was split, with 8 adults receiving the Ro-VLP vaccine and 2 adults receiving placebo. The first cohort initially consisted of 2 sentinel subjects, with at least one receiving the Ro-VLP vaccine. The remaining 8 subjects were dosed at least 24 h following the sentinel subjects. For all other cohorts, dosing occurred sequentially on the same day.

Part B of the study was conducted at a single site (the Respiratory and Meningeal Pathogens Research Unit at the Chris Hani Baragwanath Hospital, Johannesburg) in South Africa. A total of 90 healthy subjects (10 adults, 20 toddlers and 60 infants) were enrolled. One cohort of 10 adults aged 18 to 35 years was randomly assigned to receive a single intramuscular injection of the Ro-VLP vaccine (21 μg) or placebo. The adult dose cohort was split with 8 adults receiving the Ro-VLP vaccine and 2 adults receiving placebo. Two cohorts of 10 toddlers aged 12–24 months were randomly assigned to receive 2 intramuscular injections of 1 of 2 escalating dose levels of the Ro-VLP vaccine (7 μg or 21 μg) or placebo, 28 days apart. Each toddler dose cohort was split with 8 toddlers receiving the Ro-VLP vaccine and 2 toddlers receiving placebo. Three cohorts of 20 infants aged 6–10 weeks were randomly assigned to receive 3 intramuscular injections of 1 of 3 escalating dose levels of the Ro-VLP vaccine (2.5 μg, 7 μg or 21 μg) or placebo, or 2 doses of oral Rotarix, 28 days apart. Each infant dose cohort was split with 12 infants receiving the Ro-VLP vaccine, 4 infants receiving placebo and 4 infants receiving oral Rotarix. For all age cohorts, the dose levels were planned to be administered in ascending order. Progression to the next dose level was based on the absence of any vaccine-related serious adverse events in the preceding dose group 7 days post-injection. The study design is presented in Table 1.

Eligibility for the participants was assessed through medical history, clinical examination, and screening laboratory tests. Exclusion criteria included acute illness, pregnant or breastfeeding women (only adults), presence of malnutrition or any systemic disorder that would compromise the participant's health or result in non-conformance to the protocol, history of congenital abdominal disorders, intussusception or abdominal surgery (only children), known or suspected impaired immune function, immunoglobulin therapy or chronic immunosuppressant medications, known or suspected allergy to tobacco or tobacco products, a clinically significant screening laboratory value, any positive results on screening for serum hepatitis B surface antigen, hepatitis C antibody and human immunodeficiency virus (HIV) antibody, concurrent participation in another clinical trial. All adult participants were literate and provided written informed consent. Toddlers and infants were enrolled if their parents or legal guardians were literate and provided written informed consent.

The protocol and informed consent form were reviewed and approved by the Bellberry Human Research Ethics Committee (Adelaide, Australia), the Human Research Ethics Committee of the University of the Witwatersrand (Johannesburg, South Africa) and the South African Health Products Regulatory Authority (Pretoria, South Africa).

2.4. Study procedures

Prior to performing any study procedures, the investigator or designated personnel ensured that the subjects (or parents/legal guardians in the case of toddlers and infants) were given full and adequate oral and written information about the study and had signed the informed consent form. Routine laboratory tests (i.e., hematology, serum biochemistry performed according to local specifications, serology for hepatitis B virus, hepatitis C virus and HIV and coagulation/urinalysis/drugs of abuse screen (only adults)) were conducted as screening evaluations. A serum pregnancy test at screening and a urine pregnancy test 28 days after vaccination was conducted for adult female participants of child-bearing potential. Randomization took place via an interactive web response system after confirmation of inclusion and exclusion criteria before administration of the first treatment. According to a computer-generated randomization list, subjects were randomly allocated to treatment groups (Ro-VLP vaccine or placebo (or Rotarix in the case of infants)) until the target number for each dosing group was reached, and given a corresponding subject number. The eligible subjects in higher dose of each age cohort were sequentially randomized after the first week safety evaluation of the preceding dose. Adults received a single injection of the Ro-VLP vaccine or placebo to the deltoid muscle on the day of randomization (day 1); toddlers received two injections of the Ro-VLP vaccine or placebo to the anterolateral thigh on day 1 and day 29; and infants received three injections of the Ro-VLP vaccine or placebo to the anterolateral thigh on day 1, day 29 and day 57 or two oral doses of Rotarix on day 1 and day 29. Infants who received the Ro-VLP vaccine or placebo were offered 2 doses of Rotarix as a non-study vaccination after the final study visit. No follow-up was undertaken for children who received these Rotarix doses. For this group of infants, vaccination with Rotarix was completed by approximately 7 months of age. Other immunizations recommended for infants with the exception of licensed rotavirus vaccine were carried out during this study in accordance with the standard policy in South Africa. However, these immunizations were timed

Table 1
Study design.

Part	Cohort	Ro-VLP vaccine (μg)	Number of subjects (Ro-VLP vaccine/placebo)	Screening	Day 1	Day 29	Day 57	Day 85
A	Adult	7	8/2	B	X	B/F		
		21	8/2	B	X	B/F		
B	Adult	7	8/2	B	X	B/F		
	Toddler	7	8/2	B	X	B/X	B/F	
		21	8/2	B	X	B/X	B/F	
	Infant	2.5	12/4	B	X	B/X	B/X	B/F
		7	12/4	B	X	B/X	B/X	B/F
		21	12/4	B	X	B/X	B/X	B/F
		Rotarix*	12	B	X	B/X	B/F	

B: blood sampling for immunogenicity assessment.
X: administration of vaccine.
F: final study visit.
* 4 subjects at each Ro-VLP dose level administered orally with Rotarix.

such that there was a minimum of 14 days separation between administration of the study vaccines and standard of care immunizations.

Subjects were observed at the study site for 30 min after each study vaccination. According to the guidance or literatures [29–32], local reactions (injection site pain, redness, swelling, and itching) and systemic reactions (fever, decreased appetite, headache, muscle pain, chills, fatigue, nausea, malaise, and joint pain in adults, and fever, decreased appetite, irritability, vomiting, increased sleep, and decreased sleep, in toddlers and infants) were recorded in the subject diary for the first 7 days following each treatment. For example, each grade of local pain in infants is defined as follows: Mild- minor reaction when injection site is touched, Moderate- cries and protest when injection site is touched, Severe- cries when injected limb is moved, or does not move the injected limb as usual. Clinic visits took place 7 days after each vaccination, and a final in-clinic review was performed 28 days following the final vaccination. Unsolicited adverse events were recorded from randomization until the final study visit. Safety data were reviewed by two separate safety review committees (SRCs) periodically throughout the study. The SRC for Part A determined whether enrollment in Part B of the protocol could go forward after completion of enrollment of the Part A adult 21 μg group and the 7-day follow up period. The SRCs for Part B convened for the purpose of reviewing safety and tolerability data to confirm that no events meeting the stopping rule criteria occurred for any subjects in dose cohorts within the 7 days following the first Ro-VLP vaccine administration, and as such, determined that enrollment in the next dose level and/or age cohort could proceed. Actually, the dosing of the 7 μg group in toddler cohort was started 24 days after completing the first week safety evaluation of adults receiving 21 μg. Also, the dosing of the 2.5 μg group in infant cohort was commenced 7 days after finishing the first week safety evaluation of toddlers receiving 7 μg. Sera used for immunogenicity assessment were collected from all participants at baseline and 4 weeks after each vaccination. IgG and IgA titers were quantitated by standard ELISA assay techniques using human rotavirus strain 89-12 (G1P[8]) as antigen [33]. The reason why rotavirus 89-12 strain was used for the ELISA assay is that the preliminary examination using the two antigens, rotavirus 89-12 strain and Ro-VLP, demonstrated no difference in sensitivity and specificity between the two ELISAs (data not shown). Neutralizing antibodies to 89-12 (G1P[8]), DS 1 (G2P[4]), P (G3P [8]), VA70 (G4P[8]), WI61 (G9P[8]) and N632 (G12P[8]) human rotavirus strains were measured using an immunocolorimetric-based focus reduction neutralization test [34]. Titer was defined as the reciprocal serum dilution that resulted in a 60% reduction in infectious virus. Immunological measurements were performed according to the standard operating procedures of the Laboratory for Specialized Clinical Studies at the Division of Infectious Diseases, Cincinnati Children's Hospital Medical Center (Cincinnati, OH, USA).

2.5. Outcomes

The primary objective was to evaluate the safety and reactogenicity of the Ro-VLP vaccine independently in adults, toddlers and infants. The primary endpoints were proportion (expressed as a percentage) of subjects reporting vaccine-related adverse events in the first 28 days after vaccine administration, and local reactions and reactogenicity events in the first 7 days after vaccine administration for each dose level compared to the age matched pooled placebo group data. The secondary objective was to evaluate the immunogenicity profile of 2 dose levels of the vaccine in adults and toddlers and 3 dose levels of the vaccine in infants. The secondary endpoints were the proportion of subjects with anti-G1P[8] IgG seroresponses, the proportion of subjects with neutralizing antibody responses against the homologous G1P[8] rotavirus strain, and geometric mean titers (GMTs) of anti-G1P[8] IgG and neutralizing antibody responses 28 days after each immunization in adults, toddlers and infants. Seroresponses were defined as a >4-fold rise for anti- G1P[8] IgG and a >2-fold rise for anti-G1P[8] neutralizing antibody between baseline and 4 weeks after each immunization. Adjusted IgG and neutralizing antibody post-treatment titers accounted for the decay in maternal antibodies using the half-life calculated from subjects in the placebo group who had detectable baseline titers that were higher than at the post-immunization visit [35]. This adjustment value was established for each assay separately. The exploratory endpoints were proportion of infants with anti-G1P[8] IgA seroresponses and GMTs of anti-G1P[8] IgA and neutralizing antibody responses against heterologous G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] rotavirus strains 28 days after each immunization in infants. Anti-G1P[8] IgA seroresponse was defined as a >4-fold rise between baseline and 4 weeks after each immunization.

2.6. Statistical analysis

The sample size allowed for recognition of unacceptable adverse events occurring at a frequency of 15% or higher. The probability of observing at least one event among 8 subjects (adult and toddler cohorts) and 12 subjects (infant cohort) in each dose group was 73% and 86%, respectively if the true rate of that event were 15%. All subjects who received at least 1 dose of the Ro-VLP vaccine, placebo or Rotarix were assessed in the safety analysis. The frequency of vaccine-related adverse events and local/systemic reactions was summarized at the subject level; multiple occurrences of the same event within a subject were counted once in the maximum severity category (severe > moderate > mild). The immunogenicity analysis was carried out for subjects who had no major protocol violations and completed all study visits including receiving all doses of the Ro-VLP vaccine, placebo or Rotarix (per protocol population). Categorical variables are presented as frequency, proportion (%), and exact two-sided binomial (Clopper-Pearson) 95% confidential interval (CI). Numerical variables are presented as GMT and two-sided 95% CI obtained from the t-distribution on log transformed titers.

3. Results

3.1. Study population

The Australian cohort that enrolled 20 adults (12 males and 8 females) was comprised of the following: 3 Asian, 1 American Indian or Alaskan Native, and 1 Native Hawaiian or other pacific islander, and 15 Caucasian subjects. A total of 4 and 16 subjects were randomized to receive placebo and the Ro-VLP vaccine (8 each in the 7 μg and 21 μg groups), respectively. All subjects in the South African cohort that included 10 adults (5 each for male and female), 20 toddlers (6 males and 14 females) and 60 infants (29 males and 31 females) were Black (African origin). Ten adults were randomized to receive placebo (2 subjects) or the Ro-VLP vaccine 21 μg (8 subjects). Twenty toddlers were randomly assigned to receive placebo (4 subjects) or the Ro-VLP vaccine (8 each in the 7 μg and 21 μg groups). Sixty infants were randomized to receive placebo (12 subjects), the Ro-VLP vaccine (12 each in the 2.5 μg, 7 μg and 21 μg groups), or Rotarix (12 subjects). Baseline characteristics were similar across treatment groups for adults, toddlers and infants (Table 2).

All adult and toddler subjects received the intended number of immunizations; once for adults and twice for toddlers. All infant

Table 2
Baseline characteristics for adult, toddler and infant cohorts.

Characteristics	Adult			Toddler			Infant				
	Placebo (n = 6)	7 μg (n = 8)	21 μg (n = 16)	Placebo (n = 4)	7 μg (n = 8)	21 μg (n = 8)	Placebo (n = 12)	2.5 μg (n = 12)	7 μg (n = 12)	21 μg (n = 12)	Rotarix (n = 12)
Sex, n (%)											
Male	4 (66.7)	4 (50.0)	9 (56.3)	1 (25.0)	3 (37.5)	2 (25.0)	8 (66.7)	5 (41.7)	2 (16.7)	6 (50.0)	8 (66.7)
Female	2 (33.3)	4 (50.0)	7 (43.8)	3 (75.0)	5 (62.5)	6 (75.0)	4 (33.3)	7 (58.3)	10 (83.3)	6 (50.0)	4 (33.3)
Age	*<years>*			*<months>*			*<weeks>*				
mean (SD)	20.3 (2.7)	24.3 (3.6)	24.1 (2.8)	20.5 (1.3)	21.5 (1.7)	17.5 (2.6)	6.1 (0.3)	6.3 (0.7)	6.1 (0.3)	6.3 (0.9)	6.3 (0.7)
Range	18–24	20–30	20–29	19–22	19–23	13–22	6–7	6–8	6–7	6–9	6–8
Height (cm)											
mean (SD)	173.5 (7.1)	169.9 (6.6)	169.4 (5.2)	82.0 (3.9)	81.9 (4.4)	79.2 (1.8)	54.7 (1.4)	56.3 (2.2)	54.8 (1.9)	55.5 (2.2)	55.5 (3.5)
Range	165–185	160–181	161–176	78–87	78–89	77–82	51–56	52–60	52–58	52–60	50–59
Weight (kg)											
mean (SD)	63.9 (12.8)	73.7 (14.4)	65.8 (11.7)	11.3 (2.1)	10.3 (1.5)	10.0 (1.1)	5.1 (0.5)	5.0 (0.5)	4.7 (0.4)	4.6 (0.6)	5.0 (0.6)
Range	47–82	62–107	52–96	10–14	9–13	9–11	4–6	4–6	4–5	4–6	4–6
BMI (kg/m^2)											
mean (SD)	21.1 (3.3)	25.4 (3.2)	22.9 (3.3)	…	…	…	…	…	…	…	…
Range	16–25	22–33	18–31	…	…	…	…	…	…	…	…

…: no-data-collected.

subjects received the first dose. Only one subject in the 2.5 μg group did not receive the second dose. The third dose was missed in 1 subject each in the 2.5 μg and 7 μg groups. The subject disposition is represented in Fig. 1.

3.2. Safety

Subjects that received at least one dose of placebo, the Ro-VLP vaccine or Rotarix were eligible for the safety assessment. There were no deaths or serious adverse events in adults and toddlers. No deaths occurred in infants. There were 5 serious adverse events in infants: 3 subjects with bronchiolitis, and 1 subject each with bronchitis and upper respiratory tract infection. The investigator concluded that all events weren't related to the study vaccine.

Among 30 adults assessed, the most frequent solicited local reaction at the injection site was mild/moderate pain, with higher proportions seen in the 7 μg group (87.5%) and the 21 μg group (87.6%) compared to the placebo group (33.3%). One subject in the 21 μg group experienced an event of swelling with severe intensity. The most frequent solicited systemic reaction was mild/-moderate muscle pain, with higher proportions seen in the 7 μg group (37.5%) and the 21 μg group (56.3%) compared to the placebo group (16.7%). No severe solicited systemic reactions were observed (Table 3). Two vaccine-related unsolicited adverse events consisting of myalgia and injection site pruritus were observed in the 7 μg group.

Among 20 toddlers assessed, the most frequent solicited local reaction at the injection site was mild/moderate redness, with

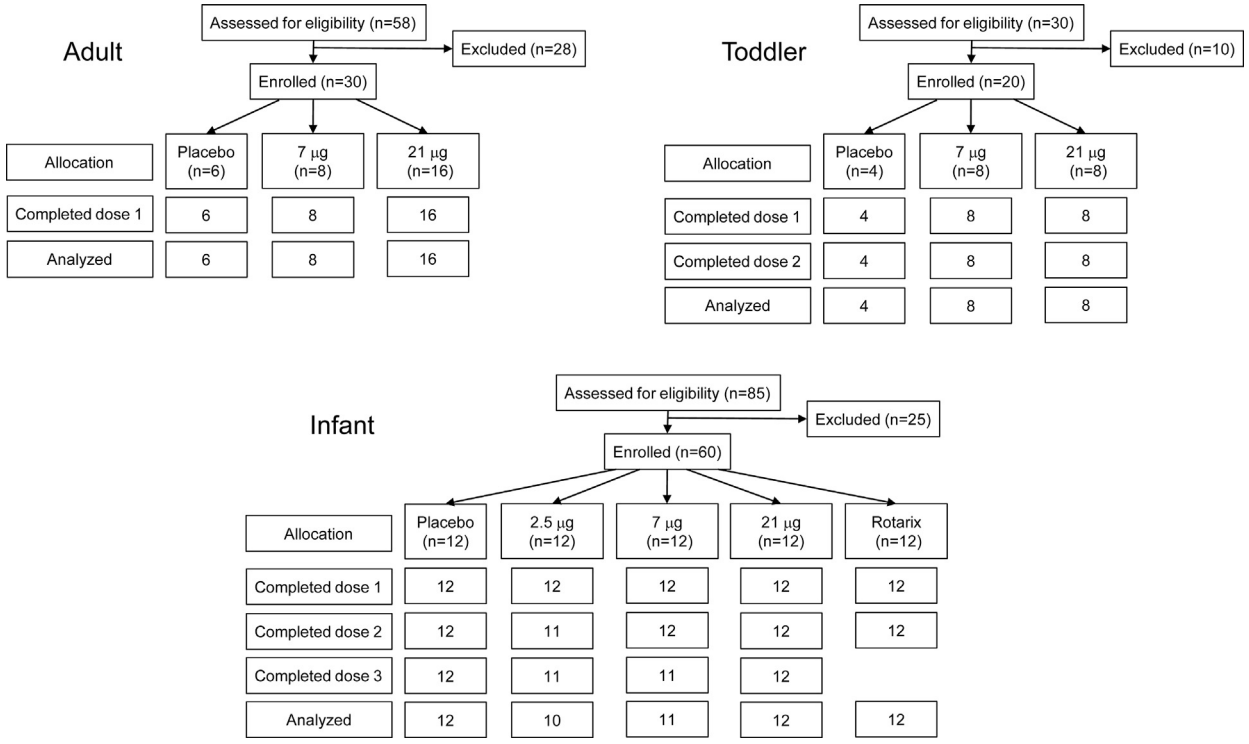

Fig. 1. Trial profile. One subject in the 2.5 μg infant group did not visit after the first dose. One subject in the 7 μg infant group did not visit after the second dose. One subject in the 2.5 μg infant group is excluded from PP population due to the subject did not receive the intended second dosage.

Table 3
Solicited local and systemic reactions in the adult group.

Symptoms	Grade	Placebo (N = 6)	7 μg (N = 8)	21 μg (N = 16)	Symptoms	Grade	Placebo (N = 6)	7 μg (N = 8)	21 μg (N = 16)
Solicited local reactions					*Solicited systemic reactions*				
Pain	mild/moderate	2 (33.3)	7 (87.5)	14 (87.6)	Fever	mild/moderate	0	0	0
	severe	0	0	0		severe	0	0	0
Redness	mild/moderate	0	0	1 (6.3)	Decreased appetite	mild/moderate	0	1 (12.5)	2 (12.5)
	severe	0	0	0		severe	0	0	0
Swelling	mild/moderate	0	0	1 (6.3)	Headache	mild/moderate	3 (50.0)	4 (50.0)	5 (31.3)
	severe	0	0	1 (6.3)		severe	0	0	0
Itching	mild/moderate	0	0	2 (12.5)	Muscle pain	mild/moderate	1 (16.7)	3 (37.5)	9 (56.3)
	severe	0	0	0		severe	0	0	0
					Chills	mild/moderate	0	2 (25.0)	1 (6.3)
						severe	0	0	0
					Fatigue	mild/moderate	2 (33.3)	1 (12.5)	6 (37.5)
						severe	0	0	0
					Nausea	mild/moderate	0	2 (25.0)	2 (12.5)
						severe	0	0	0
					Malaise	mild/moderate	0	0	1 (6.3)
						severe	0	0	0
					Joint pain	mild/moderate	1 (16.7)	0	3 (18.8)
						severe	0	0	0

. . .: no-data-collected.

higher proportions seen in the 7 μg group (50%) and the 21 μg group (37.5%) compared to the placebo group (25.0%) after the first dose; in the 7 μg group (50.0%) and the 21 μg group (25.0%) compared to the placebo group (0%) after the second dose. In addition, the proportion of mild/moderate pain was 37.5% each in the 7 μg and 21 μg groups and 0% in the placebo group after the first dose, and 62.5% in the 7 μg group, 12.5% in the 21 μg group and 25.0% in the placebo group after the second dose. No events of severe intensity were observed (Table 4). The most frequent solicited systemic reactions were decreased appetite and irritability with mild/moderate intensity, at 50.0% in the 7 μg group after the first dose, followed by mild/moderate decreased appetite, decreased sleep and increased sleep, at 50.0% in the placebo group after the first or second dose (Table 5). No severe solicited systemic reactions were observed. As for vaccine-related unsolicited adverse events, gastroenteritis (i.e., acute gastroenteritis) was observed in 1 subject in the 7 μg group and diarrhea (i.e., noninfectious diarrhea or loose stools) in 1 subject each in the 21 μg group and the placebo group.

Among 60 infants assessed, the most frequent solicited local reaction at the injection site was mild/moderate pain, with higher proportions seen in the 2.5 μg group (41.7%), the 7 μg group (75.0%) and the 21 μg group (53.3%) compared to the placebo group (16.7%) after the first dose; in the 2.5 μg group (33.3%), the 7 μg group (75.0%) and the 21 μg group (25.0%) compared to

Table 4
Solicited local reactions in the toddler and infant groups.

Symptoms	Number of doses	Grade	Toddler			Infant			
			Placebo	7 μg	21 μg	Placebo	2.5 μg	7 μg	21 μg
			(N = 4) n (%)	(N = 8) n (%)	(N = 8) n (%)	(N = 12) n (%)	(N = 12) n (%)	(N = 12) n (%)	(N = 12) n (%)
Pain	1	mild/moderate	0	3 (37.5)	3 (37.5)	2 (16.7)	5 (41.7)	9 (75.0)	7 (53.3)
		severe	0	0	0	0	1 (8.3)	0	0
	2	mild/moderate	1 (25.0)	5 (62.5)	1 (12.5)	2 (16.7)	4 (33.3)	9 (75.0)	3 (25.0)
		severe	0	0	0	0	0	0	1 (8.3)
	3	mild/moderate	. . .	. . .	. . .	3 (25.0)	3 (25.0)	3 (25.0)	3 (25.0)
		severe	. . .	. . .	. . .	0	0	0	0
Redness	1	mild/moderate	1 (25.0)	4 (50.0)	3 (37.5)	1 (8.3)	1 (8.3)	2 (16.7)	2 (16.7)
		severe	0	0	0	0	0	0	0
	2	mild/moderate	0	4 (50.0)	2 (25.0)	0	0	1 (8.3)	1 (8.3)
		severe	0	0	0	0	0	0	0
	3	mild/moderate	. . .	. . .	. . .	1 (8.3)	0	0	0
		severe	. . .	. . .	. . .	0	0	0	0
Swelling	1	mild/moderate	0	2 (25.0)	2 (25.0)	0	0	4 (33.3)	2 (16.7)
		severe	0	0	0	0	0	0	0
	2	mild/moderate	0	4 (50.0)	2 (25.0)	0	0	3 (25.0)	1 (8.3)
		severe	0	0	0	0	0	0	0
	3	mild/moderate	. . .	. . .	. . .	0	0	0	0
		severe	. . .	. . .	. . .	0	0	0	0
Itching	1	mild/moderate	0	2 (25.0)	1 (12.5)	0	2 (16.7)	0	0
		severe	0	0	0	1 (8.3)	0	0	0
	2	mild/moderate	0	0	1 (12.5)	0	0	0	0
		severe	0	0	0	0	0	0	0
	3	mild/moderate	. . .	. . .	. . .	0	0	1 (8.3)	0
		severe	. . .	. . .	. . .	0	0	0	0

Data represent number of subjects in each category and percentage calculated by using the numbers of subjects (N) for treatment group as the denominator. . . .: no-data-collected.

Table 5
Solicited systemic reactions (mild/moderate) in the toddler and infant groups.

Symptoms	Number of doses	Toddler			Infant				
		Placebo	7 μg	21 μg	Placebo	2.5 μg	7 μg	21 μg	Rotarix
		(N = 4) n (%)	(N = 8) n (%)	(N = 8) n (%)	(N = 12) n (%)	(N = 12) n (%)	(N = 12) n (%)	(N = 12) n (%)	(N = 12) n (%)
Fever	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	1 (8.3)	0
	3	...	...	...	0	0	0	1 (8.3)	...
Decreased appetite	1	2 (50.0)	4 (50.0)	0	1 (8.3)	4 (33.3)	1 (8.3)	4 (33.3)	3 (25.0)
	2	0	0	1 (12.5)	2 (16.7)	1 (8.3)	1 (8.3)	2 (16.7)	2 (16.7)
	3	...	...	...	2 (16.7)	1 (8.3)	0	1 (8.3)	...
Irritability	1	0	4 (50.0)	0	4 (33.3)	7 (58.3)	6 (50.0)	3 (25.0)	5 (41.7)
	2	0	3 (37.5)	0	2 (16.7)	3 (25.0)	3 (25.0)	4 (33.3)	2 (16.7)
	3	...	...	...	2 (16.7)	3 (25.0)	4 (33.3)	1 (8.3)	...
Decreased activity	1	0	2 (25.0)	1 (12.5)	0	6 (50.0)	1 (8.3)	3 (25.0)	4 (33.3)
	2	0	1 (12.5)	0	1 (8.3)	0	3 (25.0)	3 (25.0)	1 (8.3)
	3	...	...	...	1 (8.3)	1 (8.3)	2 (16.7)	1 (8.3)	...
Vomiting	1	0	1 (12.5)	0	1 (8.3)	1 (8.3)	1 (8.3)	4 (33.3)	2 (16.7)
	2	0	0	1 (12.5)	2 (16.7)	3 (25.0)	1 (8.3)	3 (25.0)	3 (25.0)
	3	...	...	...	2 (16.7)	0	1 (8.3)	1 (8.3)	...
Decreased sleep	1	2 (50.0)	2 (25.0)	1 (12.5)	3 (25.0)	5 (41.7)	1 (8.3)	3 (25.0)	4 (33.3)
	2	2 (50.0)	1 (12.5)	0	2 (16.7)	2 (16.7)	2 (16.7)	4 (33.3)	2 (16.7)
	3	...	...	...	0	1 (8.3)	2 (16.7)	1 (8.3)	...
Increased sleep	1	2 (50.0)	3 (37.5)	1 (12.5)	2 (16.7)	5 (41.7)	2 (16.7)	2 (16.7)	4 (33.3)
	2	0	0	2 (25.0)	0	1 (8.3)	3 (25.0)	4 (33.3)	3 (25.0)
	3	...	...	...	1 (8.3)	0	2 (16.7)	2 (16.7)	...

Data represent number of subjects in each category and percentage calculated by using the numbers of subjects (N) for treatment group as the denominator. ...: no-data-collected.

the placebo group (16.7%) after the second dose; and 25.0% each in the 2.5 μg, 7 μg and 21 μg groups and placebo group after the third dose. A trend was noted of higher proportions of subjects with solicited local reactions such as pain, redness and swelling after the first dose compared to the second or third dose. Few solicited local reactions of severe intensity were observed: 1 event of pain each in the 2.5 μg group after the first dose and the 21 μg group after the second dose, and 1 event of itching in the placebo group after the first dose (Table 4). The most frequent solicited systemic reaction was mild/moderate irritability seen in the 2.5 μg group (58.3%), the 7 μg group (50.0%), the 21 μg group (25.0%), the Rotarix group (41.7%) and the placebo group (33.3%) after the first dose. Other solicited systemic reactions were dispersed across categories and treatment groups with no noticeable trend (Table 5). No severe solicited systemic reactions were observed. One vaccine related adverse event of conjunctivitis was observed in the 2.5 μg group.

3.3. Immunogenicity

All 30 adults and 20 toddlers included in the safety assessment were eligible for the per-protocol immunogenicity analysis. The proportions of adults with anti G1P[8] IgG seroresponses defined as a >4-fold rise in titer between baseline and 4 weeks after the last dose were 75.0% (6 of 8 adults) in the 7 μg group and 50.0% (8 of 16 adults) in the 21 μg groups. Seroresponses defined as a >2-fold rise in neutralizing antibody titer to G1P[8] were observed in 12.5% (1 of 8 adults) in the 7 μg group and 25.0% (4 of 16 adults) in the 21 μg group. The proportions of toddlers with anti G1P[8] IgG seroresponses after the second dose were 62.5% (5 of 8 toddlers) in the 7 μg group and 87.5% (7 of 8 toddlers) in the 21 μg group. Seroresponses in neutralizing antibody to G1P[8] after the second dose occurred in 75.0% (6 of 8 toddlers) in the 7 μg group and 50.0% (4 of 8 toddlers) in the 21 μg group. Both adult and toddler placebo groups showed no increase in anti-G1P[8] IgG and neutralizing antibody responses.

Among 60 infants included in the safety assessment, 57 (including 12 subjects in the placebo group, 10 subjects in the 2.5 μg group, 11 subjects in the 7 μg group, 12 subjects in the 21 μg group, and 12 subjects in the Rotarix group) who completed all study visits including receiving all intended doses of placebo, the Ro-VLP vaccine or Rotarix, with no major protocol violations, were eligible for the per-protocol immunogenicity analysis.

The Ro-VLP vaccine induced a steady rise in IgG GMTs against homologous G1P[8] rotavirus 4 weeks after the second dose, with the immune response becoming more prominent after the third dose (Fig. 1). No clear differences among the Ro-VLP vaccine groups were observed for anti G1P[8] IgG GMTs. The proportions of infants with adjusted anti G1P[8] IgG seroresponses defined as a >4-fold rise in titer between baseline and 4 weeks after the third dose were 70.0% in the 2.5 μg group, 100% in the 7 μg group and 91.7% in the 21 μg group. No seroresponses were observed in the placebo group 4 weeks after the third dose, while 2 (16.7%) subjects in the Rotarix group showed a seroresponse 4 weeks after the second dose (Table 6).

An increase in anti-G1P[8] IgA GMTs was observed in the 7 μg and 21 μg groups as well as the Rotarix group 4 weeks after each dose (Fig. 2). The proportions of infants with anti G1P[8] IgA seroresponses based on a >4-fold rise in titer between baseline and 4 weeks after the last dose were 63.6% in the 7 μg group, 75.0% in the 21 μg group and 41.7% in the Rotarix® group. The placebo and 2.5 μg groups showed no increase in anti-G1P[8] IgA response (Table 6).

Four weeks after the third dose, the GMTs of neutralizing antibody responses against homologous G1P[8] rotavirus were higher in all Ro-VLP vaccine groups than in the placebo group. The Ro-VLP vaccine groups demonstrated an increase in neutralizing antibody titer after an initial decline, whereas the placebo group showed a continuous decline below the levels of neutralizing antibody titer displayed in the Ro-VLP vaccine groups (Fig. 2). About half of the subjects that received the Ro-VLP vaccine demonstrated seroresponses for adjusted anti-G1P[8] neutralizing antibody titer defined as a >2-fold rise between baseline and 4 weeks after the third dose, at 30.0% in the 2.5 μg group, 45.5% in the 7 μg group and 58.3% in the 21 μg groups. It's worth noting that the seroresponses were observed in 86.7% (13 of 15 subjects) of infants with low baseline titers (<50) across the Ro-VLP vaccine groups. The

Table 6
Seroresponses of IgG, IgA and neutralizing antibody against human rotavirus 89-12 (G1P[8]) in the per-protocol infant population.

	Pre-vaccination GMT (95% CI)	Post-vaccination GMT (95% CI)	Seroresponse, unadjusted n (%, 95% CI)	Seroresponse, adjusted n (%, 95% CI)
Anti-G1P[8] IgG				
Placebo (n = 12)	266 (148–478)	68 (39–118)	0 (0–26.5)	0 (0–26.5)
2.5 μg (n = 10)	239 (118–485)	829 (424–1620)	4 (40.0%; 12.2–73.8)	7 (70.0%; 35–93)
7 μg (n = 11)	300 (174–519)	1119 (766–1633)	5 (45.5%; 16.7–76.6)	11 (100%; 72–100)
21 μg (n = 12)	250 (146–430)	1237 (795–1924)	6 (50.0%; 21.1–78.9)	11 (91.7%; 62–100)
Rotarix (n = 12)	191 (111–327)	149 (94–236)	0 (0–26.5)	2 (17%: 2–48)
Anti-G1P[8] IgA				
Placebo (n = 12)	4	4	0 (0–26.5)	. . .
2.5 μg (n = 10)	5 (3–8)	8 (4–16)	1 (10%; 0.3–44.5)	. . .
7 μg (n = 11)	5 (3–7)	36 (14–94)	7 (63.6%; 30.8–89.1)	. . .
21 μg (n = 12)	4 (3–5)	24 (14–43)	9 (75.0%; 42.8–94.5)	. . .
Rotarix (n = 12)	5 (3–9)	25 (7–87)	5 (41.7%; 15.2–72.3)	. . .
Anti-G1P[8] neutralizing antibody				
Placebo (n = 12)	35 (14–89)	9 (6–14)	0 (0–26.5)	1 (8.3%; 0.2–38.5)
2.5 μg (n = 10)	57 (21–156)	30 (13–69)	2 (20.0%; 2.5–55.6)	3 (30.0%; 6.7–65.2)
7 μg (n = 11)	73 (42–128)	47 (16–137)	3 (27.3%; 6.0–61.0)	5 (45.5%; 16.7–76.6)
21 μg (n = 12)	39 (20–76)	47 (18–119)	4 (33.3%; 9.9–65.1)	7 (58.3%; 27.7–84.8)
Rotarix (n = 12)	26 (13–53)	19 (10–38)	1 (8.3%; 0.2–38.5)	3 (25.0%; 5.5–57.2)

Post-vaccination GMT and seroresponses were determined using serum titers 4 weeks after the last dose (the placebo and the Ro-VLP groups: the third dose, Rotarix: the second dose). IgG and neutralizing antibody titers after vaccination were adjusted for decay in maternal antibodies using the half-life calculated from subjects in the placebo group who had detectable baseline titers that were higher than at the after-vaccination visit and established for each assay separately.

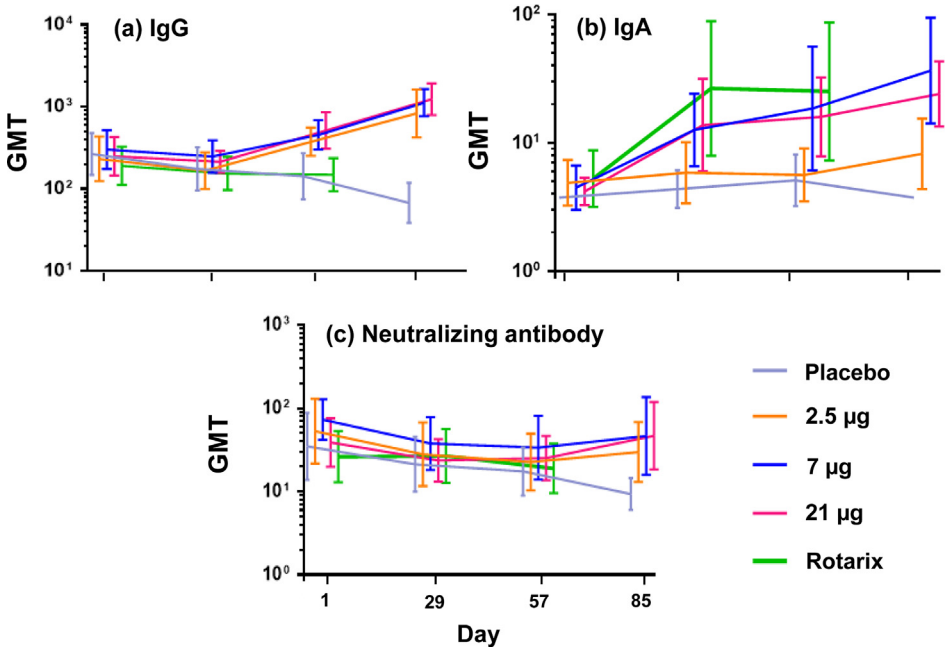

Fig. 2. Serum antibody responses against human rotavirus 89-12 (G1P[8]) in the per-protocol infant population. (a) IgG, (b) IgA, (c) neutralizing antibody. The antibody titers were measured at baseline (Day 1), 4 weeks after the first dose (Day 29), the second dose (Day 57) and the third dose (Day 85). Data represent unadjusted geometric mean titer (GMT). Error bars show 95% CI.

proportions of seroresponses were 8.3% in the placebo group 4 weeks after the third dose and 25.0% in the Rotarix group 4 weeks after the second dose (Table 6).

Neutralizing antibody titers against five heterologous rotaviruses, G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8], showed a continuous decline throughout the immunogenicity assessment period in all Ro-VLP vaccine groups as well as the placebo group, suggesting that the Ro-VLP vaccine did not induce a heterotypic neutralizing antibody response. There were also no clear differences in neutralizing antibody responses against all five rotaviruses between the placebo group and the Rotarix group 4 weeks after the second dose.

4. Discussion

The Ro-VLP vaccine was well-tolerated with few mild unsolicited adverse events in all age cohorts tested, and demonstrated an encouraging immunogenicity in infants. Infants in the Ro-VLP vaccine groups showed a higher frequency of solicited local or systemic reactions such as pain and swelling at the injection site and irritability compared to those in the placebo group, while most symptoms were of mild or moderate intensity. The incidence of reactogenicity events after immunization with the Ro-VLP vaccine was similar to those reported for licensed hexavalent pediatric vaccines such as Hexyon® and Infanrix hexa™ [36]. Furthermore, the

safety and reactogenicity profiles in adults of the plant-made influenza vaccine candidate manufactured using the same technology as that of the Ro-VLP vaccine was similar to those reported for commercially available inactivated influenza vaccines [37].

A robust serum IgG response against homologous G1P[8] rotavirus was elicited by immunization with the Ro-VLP vaccine. Most infants in the 7 μg and 21 μg groups achieved seroresponses 4 weeks after the third dose. At the same time, the proportion of anti-G1P[8] IgA seroresponses was lower than that of IgG seroresponses. The lower serum IgA response is not surprising given that the Ro-VLP vaccine was administered intramuscularly. It has been reported that intramuscular injections of live or inactivated rotavirus in rabbits can induce protective immunity that may be mediated by anti-rotavirus IgG in the intestine [38]. Additionally, results from a non-human primate model demonstrated that passively transferred sera with a high rotavirus-specific IgG titer suppresses or delays rotavirus infection [39]. These findings suggest that antigen-specific serum IgG induced by parenteral immunization with rotavirus vaccine has the potential to contribute to protection from rotavirus infection through transudation or permeation into the intestinal lumen.

The Ro-VLP vaccine induced a neutralizing antibody response against homologous G1P[8] rotavirus with lower titer than the specific IgG response. However, it is noteworthy that the neutralizing antibody titers 4 weeks after the third dose of 7 μg or 21 μg rebounded to the baseline level. This indicates that infants successfully replaced all maternally-derived antibodies that were lost from natural waning. In the phase 1/2 study of a parenteral trivalent P2-VP8 subunit vaccine that is currently being developed as a non-replicating rotavirus vaccine, the neutralizing antibody responses to all three rotavirus strains tested have been shown to follow a similar time course as that observed for the Ro-VLP vaccine. Moreover, a reduction in fecal shedding was confirmed after a challenge of oral Rotarix [40], which suggests that the neutralizing antibody titers detected 4 weeks after the third dose of the Ro-VLP vaccine might reach a level sufficient to exert local protection at the gut surface.

Heterotypic immunity, which has been observed with live oral rotavirus vaccines [41], was not elicited by immunization with the Ro-VLP vaccine. In an analysis of neutralizing monoclonal antibodies derived from adult humans, immunoglobulins (Igs) directed to VP5, the stalk region of the rotavirus attachment protein VP4, have represented more commonly heterotypic responses than Igs against the outer shell protein VP7, or VP8, the cell-binding region of VP4 [42]. Therefore, Ro-VLP composed of VP7, VP6, and VP2, may have little potential to induce cross-reactive immunity given its protein composition. Considering that G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8], have been identified as globally circulating rotavirus strains [7], the development of a multivalent Ro-VLP vaccine that is effective to these six rotavirus strains would be desirable.

Rotarix played an important role as an internal control in this clinical trial enabling determinations of reactogenicity, tolerability and immunogenicity to be made compared to an approved vaccine in infants. This study demonstrated the importance of such a control in vaccine trials, because even for Rotarix, the antibody levels may appear different from that reported from other clinical trials, with such differences most likely reflecting the difficulty in attempting to account for absolute differences in measured antibody responses between historical publications that may be attributable to differences in vaccine lots, assay methodology, and the study population. However, the use of Rotarix does permit the antibody responses observed to Ro-VLP to be placed in context with those elected by Rotarix which has been established to be efficacious against disease caused by some serotypes of rotavirus.

A number of limitations exist in our study. The number of subjects evaluated was small in all three age cohorts because the study was primarily designed to collect information on safety and immunogenicity to determine whether further exploration of this vaccine platform is warranted. Furthermore, the purpose of including adult and toddler cohorts was mainly to confirm that there are no serious concerns regarding safety and tolerability before proceeding to the infant cohort, the target population for the Ro-VLP vaccine. There were no apparent differences in the immune responses of infant subjects between the 7 μg and 21 μg groups; however, further studies to examine the dose–response relationship will be necessary to explore the optimal dosage of plant-derived rotavirus VLP vaccines. Immunogenicity assessment in infants was completed 4 weeks after the third dose because it was necessary from an ethical standpoint to inoculate infants who had received the Ro-VLP vaccine or placebo with Rotarix after the final study visit. Therefore, this study does not provide any information on how long the immune responses induced by the Ro-VLP vaccine persist. Based on several pieces of evidence from a number of developing countries [43,44], questions about the durability of currently licensed rotavirus vaccines beyond the first year of life have been raised [45]. The most suitable vaccination regimen, including prime- boost immunization with plant-derived rotavirus VLP vaccines, needs to be further investigated with future clinical studies. Cell-mediated immunity was not investigated as an immunogenicity endpoint in this study. Evidence from human studies suggests that rotavirus-specific T cells are important for developing protective immune responses [46–48]. A plant-derived virus-like particle influenza vaccine candidate manufactured using the same technology as the Ro-VLP vaccine has been shown to induce antigen-specific $CD4^+$ T cell responses in adult humans [26,37]. Given that no correlate of protection has been identified for rotavirus disease [49], cellular immunity might be a subject of investigation in the future of clinical development for plant-derived rotavirus VLP vaccines.

The development of a non-replicating rotavirus vaccine has great potential to overcome a number of the outstanding issues of currently marketed live-attenuated oral rotavirus vaccines. The results of this study support further development of plant-derived rotavirus VLP vaccines that are effective against virus strains causing clinically relevant disease in infants and young children worldwide.

Author contributions

NK, MKR, CB, ST, BDF, and NT conceived and designed the study. AK, SM, and TMP acquired clinical data. MM acquired immunogenicity data. YUK and YOK analyzed the data. MD and MMC produced the Ro-VLP vaccine. NK, MKR, CB, BDF, and NT interpreted the data. NK and NT drafted the manuscript. All authors critically revised the manuscript and approved of the final version for submission.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: MM (Monica McNeal) has laboratory service agreements with Merck &Co., Inc, outside of the submitted work. No other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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